AD_____

Award Number: DAMD17-98-1-8605

TITLE: Enhancing Effect of Radiation Therapy Using Non-Steroidal Anti-Inflammatory Agents

PRINCIPAL INVESTIGATOR: C. Norman Coleman, M.D.

CONTRACTING ORGANIZATION: National Institute of Health
Bethesda, Maryland 20892-1904

REPORT DATE: April 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Annual (1 Oct 00 - 31 Mar 01)

4. TITLE AND SUBTITLE
Enhancing Effect of Radiation Therapy Using Non-Steroidal Anti-Inflammatory Agents

5. FUNDING NUMBERS
DAMD17-98-1-8605

6. AUTHOR(S)
C. Norman Coleman, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
National Institutes of Health
Bethesda, Maryland 20892-1904

E-Mail : ccoleman@mail.nih.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Our earlier studies demonstrated that ibuprofen sensitizes prostate carcinoma cells to radiation *in vitro* and *in vivo*. The cytotoxic and radiosensitizing effects of ibuprofen were seen at much higher concentration than that required to inhibit prostaglandin synthesis. To understand the molecular mechanisms involved in radiosensitization we examined the effects of ibuprofen on several potential cellular targets including COX-2 and transcription factor NFkB. COX-2 was constitutively expressed in PC3 cells and was further upregulated by NSAIDs. Ibuprofen inhibited constitutive as well as cytokine- or raditation-activated NFkB in prostate cancer cells. Currently we are evaluating the effect of ibuprofen on hypoxia-induced expression of the angiogenic factor HIF-1 α and the subsequent VEGF secretion. HIF-1α protein is constitutively expressed in prostate cancer cell lines under normoxic condition and is significantly upregulated by hypoxia. Ibuprofen inhibited HIF-1α under normoxic condition but was less effective under hypoxic condition. Ibuprofen inhibited VEGF protein under normoxic and hypoxic condition. We are also evaluating the effects of other NSAIDs that selectively inhibit COX-2 and have proven to be less toxic in clinic. The results from this research project can be directly translated into the clinic with a potential to improve local tumor control, to reduce toxicity and increase overall survival.

14. SUBJECT TERMS	15. NUMBER OF PAGES
COX-2, NSAIDs, radiation, HIF, VEGF, NFkB	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of contents

Introduction:

Our earlier studies demonstrated that ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), sensitizes prostate carcinoma cells to radiation *in vitro* and *in vivo* (1, 2). *In vivo*, the response was found to be superior to that of anti-androgens (2). The accepted hypothesis for mechanisms underlying the actions of NSAIDs is that cyclooxygenase enzymes are inhibited and prostaglandin (PG) synthesis is blocked. There are two isoforms of cyclooxygenase, COX-1 and COX-2, and nonspecific NSAIDs inhibit both isoforms. COX-1 is constitutively expressed in most tissues and produces prostaglandins that are essential for homeostasis of platelets, kidney and gut mucosa. Inhibition of COX-1 therefore results in undesirable toxicity in the patients. COX-2 is an inducible enzyme, induced in response to stress stimuli including inflammatory cytokines, growth factors and hypoxia. Several reports indicate that COX-2 is constitutively expressed in a variety of human tumors (3). Although the COX isoforms are inhibited by NSAIDs at concentrations in submicromolar range (4), the cytotoxic effects of NSAIDs including induction of apoptosis, are seen at much higher doses (1, 5-8). Therefore, it appears that mechanisms other than the inhibition prostaglandin synthesis are involved in growth inhibition and anti-tumor effects of NSAIDs. Further evidence for this comes from the observations that NSAIDs induce apoptosis and cytotoxicity even in cells that lack COX transcripts (9, 10) and chronic exposure to NSAIDs at cytotoxic concentrations *increases* COX-2 transcripts and protein in a variety of cell types (6-8). Moreover, colon carcinoma cell growth is inhibited by both sulindac sulfide, a PG synthesis inhibitor, and sulidac sulfone, a derivative that essentially lacks PG synthesis-inhibitory activity (5). NSAIDs thus appear to have multiple cellular targets.

As proposed in our grant application, we are investigating the effect of NSAIDs on two potential non COX-2 molecular targets. 1) NFkB, the key transcription factor in cytokine regulation that also has anti-apoptotic properties (11) and 2) HIF-1, the hypoxia inducible transcription factor, which activates vascular endothelial growth factor (VEGF) gene, and regulates tumor angiogenesis in hypoxic tumor environment. Moreover, keeping up to date with the recent developments in COX-2 field (3), studies on COX-2 have been continued. COX-2 specific inhibitors appear to be particularly suitable to use in the clinic, as they are much less toxic than the nonspecific NSAIDs. COX-2 specific inhibitors also reportedly inhibit tumor angiogenesis and thus may be of significant importance in cancer therapy.

Research Accomplishments:

1. Effect of NSAIDs on COX (*Task 2*)**:**

a) COX-2 protein: Since the cytotoxic effects of NSAIDs were observed at higher concentrations, the effect of NSAIDs on COX-2 protein was studied by western blot analysis at those concentrations. In addition to the non-specific NSAIDs ibuprofen and diclofenac, we also studied the effect of COX-2 specific inhibitors NS 398, niflumic acid and nimesulide, and 5-lipoxygenase inhibitors MK886 and Rev 5901 on COX-2 protein levels in prostate cancer cells. COX-2 protein is constitutively expressed in PC3 cells. Interestingly, COX-2 protein level *increased* when cells were treated with non-specific NSAIDs, COX-2 inhibitor NS-398 as well as lipoxygenase inhibitors (***Appendix, Fig. 1***). The increase was evident at 6h and persisted up to 48h.

b) COX-2 protein, NSAIDs in combination with radiation or hypoxia:
Radiation: No significant changes in COX-2 protein level were observed at 24h in PC3 cells treated with 8-10 Gy radiation. A transient increase in COX-2 protein at 6h was reported in PC3 cells irradiated at 5 and 15 Gy (12). Ibuprofen increased COX-2 in irradiated cells as well (**Figure 1**).
Hypoxia: Hypoxia increased COX-2 protein, particularly, if cells were grown in low serum media. Ibuprofen and NS 398 increased COX-2 protein levels even under hypoxic condition (**Figure 2**).

Figure 1. COX-2 protein levels in PC3 cells treated with ibuprofen and radiation. PC3 cells constitutively express COX-2 protein. COX-2 levels significantly *increased* by ibuprofen (2mM) in non-irradiated and irradiation cells.

Figure 2. COX-2 protein levels in PC3 cells treated with NS 398 (100 μM), a COX-2 specific inhibitor, under normoxic and hypoxic conditions. Hypoxia increased COX-2 protein. COX-2 levels significantly *increased* by NS 398 under normoxic and hypoxic conditions. NS 398 treatment was in 0.1% media.

Figure 1

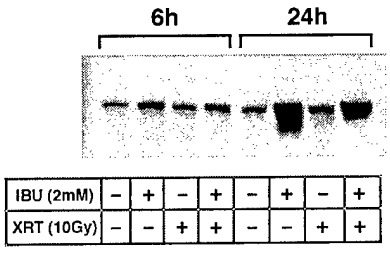
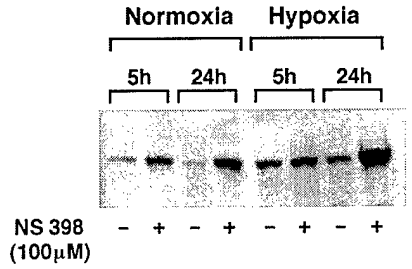


Figure 2



DU 145 and LNCaP cells did not express COX-2 protein. Also no induction of COX-2 protein was detected in DU-145 and LNCaP cells following treatment with NSAIDs, radiation or hypoxia.

2. Radiosensitization by COX-2 and lipoxygenase inhibitors:

Our earlier studies indicated that ibuprofen enhanced radiation cytotoxicity (1). We further studied the effect of other NSAIDs, COX-2 inhibitors and 5-lipoxygenase inhibitors on clononogenic survival in irradiated PC3 cells (*Task 2*). Preliminary data indicated that 5-lipoxygenase inhibitors were cytotoxic at micromolar concentrations and also enhanced radiation cytotoxicity. However, COX-2 inhibitors were less cytotoxic and did not enhance radiation cytotoxicity at the concentrations used in these experiments (**Figure 3**).

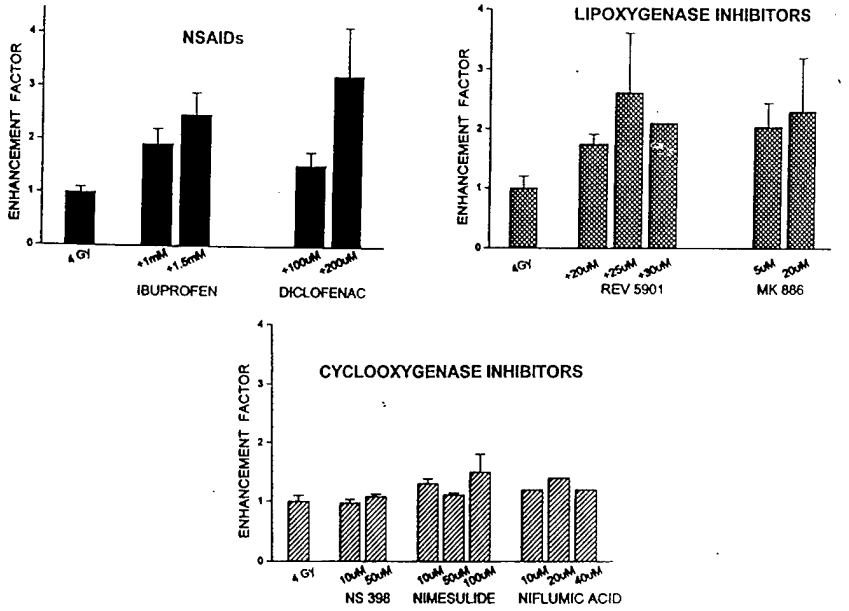


Figure 3. PC3 cells were treated with various eicosanoid inhibitors, irradiated (4 Gy) and plated for clonogenic cell survival assay after 24h. NSAIDs (ibuprofen and diclofenac), and 5-lipoxygenase inhibitors (REV 5901 and MK886) enhanced radiation-induced cytotoxicity. COX-2 inhibitors (NS 398, nimesulide and niflumaic acid) were not as effective at the concentrations used in this study.

3. Effect of NSAIDs on angiogenic factors

Development of new blood vessels is a critical requirement for tumor growth (13,14). It is well known that tumor cells produce a variety of angiogenic factors including prostaglandins, basic fibroblast growth factor (bFGF), transforming growth factor-B (TGF-B) and vascular endothelial growth factor (VEGF). COX-2 is also implicated in the regulation of tumor angiogenesis (15) although the mechanisms involved in this regulation are not fully understood. In addition, tumor angiogenesis is influenced by several other factors including oncogenic transformations and tumor hypoxia (16,17).

a) Hypoxia-inducible transcription factor (HIF-1) (*Task 3-d*)

Due to the high rate of cell proliferation, tumor vasculature is often unable to maintain a normal level of oxygenation resulting in hypoxic areas within the tumor. Adaptation to hypoxia is a major step in tumor progression (17). Hypoxia-inducible factor, HIF-1, is a transcription factor that plays a crucial role to maintain O_2 homeostasis. Most common human cancers overexpress HIF-1. Activation of various oncogenes or inactivation of tumor suppressor genes can also result in the overexpression of HIF-1α (17). Under hypoxic conditions, HIF-1 protein level is rapidly upregulated. HIF-1 is translocated to the nucleus where it activates the transcription of several genes including erythropoietin, vascular endothelial growth factor, glucose transporters and glycolytic enzymes. The activation of HIF-1 α thus increases O_2 delivery and provides metabolic adaptation under reduced O_2 conditions.

HIF-1 is a heterodimer consisting of two subunits HIF-1α and HIF-1β. Both subunits contain amino-terminal basic-helix-loop-helix-PAS (bHLH-PAS) domains that are required for dimerization and DNA binding. While HIF-1α is the specific and O_2-regulated subunit of HIF-1, HIF-1β can dimerize with several other different bHLH-PAS proteins (17). When cells are exposed to hypoxia HIF-1α protein levels increase dramatically in the absence of any change in mRNA levels, suggesting that HIF-1α is primarily regulated at the level of protein synthesis and degradation. Under normoxic conditions HIF-1α is polyubiquitinated indicating its degradation via the ubiquitin-proteasome pathway (18,19). Under hypoxic conditions HIF-1α escapes ubiquitination, resulting in an increase in protein levels, induced nuclear import, dimerization with HIF-1β and ultimately target gene activation.

Based on the observations that the majority of human tumors are hypoxic, hypoxia induces angiogenesis and angiogenesis is inhibited by NSAIDs, we hypothesized that NSAIDs may inhibit the HIF-1 transcription factor and thereby inhibit the VEGF secretion by tumor cells. A preliminary study indicated that ibuprofen inhibited VEGF mRNA in prostate cancer cells. We are currently evaluating the effects of ibuprofen and NS 398 on the hypoxia inducible transcription factors and the subsequent VEGF secretion in PC3 prostate cancer cells.

Work in progress: HIF-1α

Prostate carcinoma cell lines were treated with ibuprofen and subjected to hypoxia by gassing 5% CO2 in nitrogen. Cell extracts or nuclear and cytoplasmic extracts were prepared at various time points and HIF-1α protein was analyzed by western blot analysis.

1. Prostate cancer cells (PC3, DU-145, LNCaP) express HIF-1α under normoxic condition. Hypoxia increases HIF-1α protein level. HIF-1α protein remains elevated even at 24h. Ibuprofen inhibits HIF-1α under normoxic condition but is less effective under hypoxic condition (**Figure 5**).

2. COX-2 inhibitor NS 398 also inhibited HIF-1α under normoxic and hypoxic condition when cells are treated in 0.1% media (**Figure 6**).

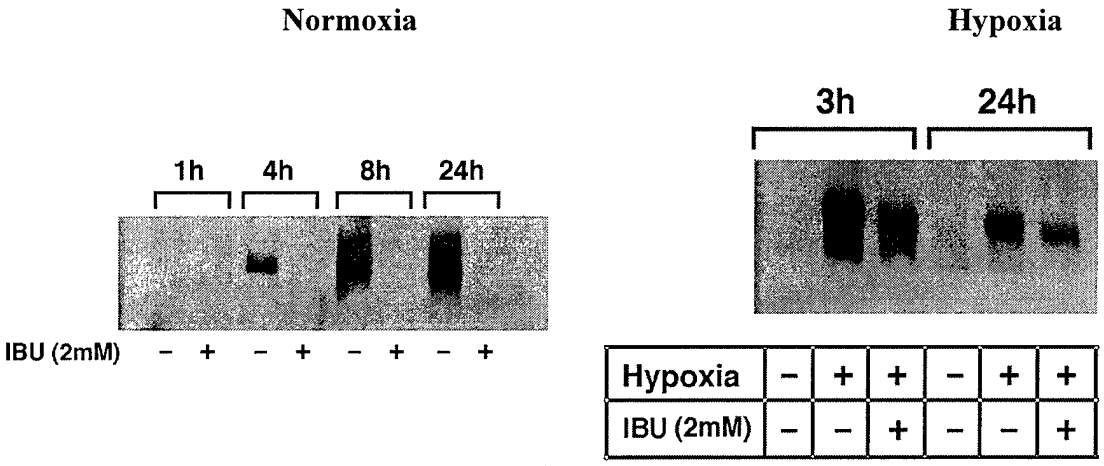


Figure 5. Inhibition of HIF-1α by ibuprofen in PC3 cells. Ibuprofen was more effective under normoxic conditions than under hypoxic conditions.

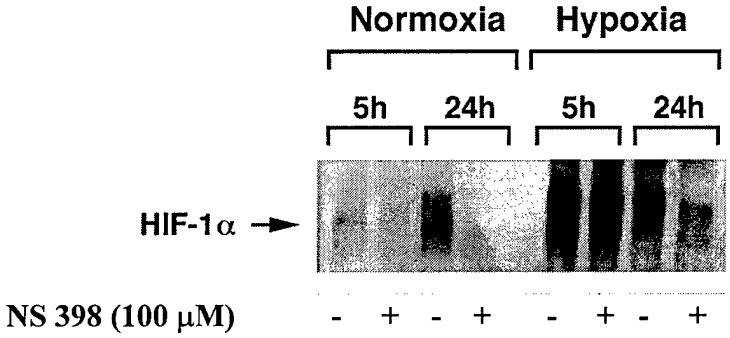


Figure 6. Inhibition of HIF-1α by NS 398, a COX-2 specific inhibitor, in PC3 cells under normoxic and hypoxic conditions. NS 398 was more effective under normoxic than hypoxic conditions.

b) Vascular endothelial growth factor (VEGF) (*Task 1, Task 3-d*):

Under hypoxic condition HIF-1α activates the transcription of VEGF, a paracrine proangiogenic growth factor. VEGF mRNA is markedly upregulated in the majority of human tumors whereas the mRNAs for the receptors Flt-1 and KDR are upregulated in tumor endothelial cells (20,21). VEGF promotor region contains hypoxia response element. Additional binding sites for other transcriptional factors such as AP-1, AP-2 and Sp1 are also present in the VEGF promoter region. These transcriptional factors are required for transcriptional activation of VEGF gene by growth factors, cytokines and MAP kinases under normoxic condition.

We studied the effect of NSAIDs on VEGF secretion in culture media under normoxic and hypoxic conditions, by ELISA assay. Ibuprofen inhibited VEGF secretion by PC3 cells in the culture media under hypoxic condition but not under normoxic conditions (**Figure 7**). These

results suggest that VEGF may be regulated by different mechanisms under hypoxic and normoxic conditions.

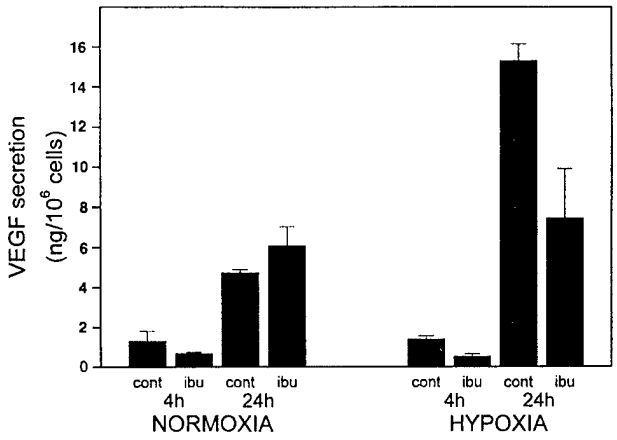


Figure 7. The concentration of VEGF in conditioned media was determined by ELISA and normalized to the cell number in the flask. Hypoxia induced ~ 3fold increase in the amount of VEGF secreted. Ibuprofen inhibited VEGF, however, at 24h, the inhibition persisted only in hypoxic cells.

Key Research Accomplishments:

We have identified novel cellular targets of ibuprofen.

1) COX-2: Low concentrations of NSAIDs inhibited prostaglandin PGE2 synthesis, however, chronic treatment with higher concentrations of NSAIDs resulted in upregulation of COX-2 protein.
2) NFkB, IKK-kinase: Ibuprofen inhibited constitutive and activated NFkB and IKK-α in prostate cancer cells.
3) HIF-1α : Ibuprofen inhibited HIF-1α in prostate cancer cells. Ibuprofen was more effective under normoxic condition as compared to hypoxic condition.
4) VEGF: Ibuprofen inhibited VEGF protein.

Reportable Outcomes:

Publication:

S. T. Palayoor, M. Y. Youmell, S. K. Calderwood, C. N. Coleman and B. D. Price, Constitutive activation of IKB-kinase a and NFkB in prostate cancer cells is inhibited by ibuprofen. Oncogene, 18, 7389-7394, 1999.

Abstracts:

S. T. Palayoor, S. K. Calderwood, E. A. Bump and C. Norman Coleman, Role of eicosanoid inhibitors in radiosensitization of PC3 prostate carcinoma cells. 47th Radiation Research society Meeting, 2000, Albuquuerque, NM.

Conclusions:

Ibuprofen, a non-steroidal anti-inflammatory agent enhanced the effects of radiation in prostate cancer cells *in vitro* and *in vivo*. We are currently investigating the cellular targets of ibuprofen. We used a panel of established human prostate cancer cell lines in this study and found COX-2, NFkB and HIF-1 to be constitutively activated in some. In general, these proteins are known to facilitate the tumor cell survival and spread. For example, the activation of NFkB increases the expression of antiapoptotic proteins. COX-2 and HIF-1 are known to promote tumor angiogenesis and metastasis. In addition, HIF-1 activates several other genes that help tumor cells to metabolically adapt and survive in unfavorable hypoxic microenvironment. These changes in tumor cells also make them resistant to the standard anti-tumor therapy. NSAIDs may prove to be valuable drugs for treatment of tumors as ibuprofen effectively interfered with these targets.

References

1. Palayoor, S.T.,et al., Combined antitumor effect of radiation and ibuprofen in human prostate carcinoma cells. Clin Cancer Res, 1998. **4**(3): p. 763-71.
2. Teicher, B.A., et al., Signal transduction inhibitors as modifiers of radiation therapy in human prostate carcinoma xenografts. Radiat Oncol Invest, 1996. **4**: p. 221-30.
3. Fosslien E., Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Critical Rev in Clin Lab Sci, 2000. **37**(5): p 431-502.
4. Meade, E.A., et al., Differential inhibition of prostaglandin endoperoxidase synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J Biol Chem, 1993. 268: p. 6610-6614.
5. Piazza, G.A., et al., Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. Cancer Res, 1995. **55**(14): p. 3110-6.
6. Lu, X., et al., Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7961-5.
7. Meade, E.A., et al., Peroxisome proliferators enhance cyclooxygenase-2 expression in epithelial cells. J Biol Chem, 1999. **274**(12): p. 8328-34.
8. Simmons, D.L., et al., Induction of an acetttaminophen-sensitive cyclooxygenase with reduced sensitivity to nonsterodal antiinflammatory drugs. Proc Natl Acad Sci, 1999. **96**: p. 3275-3280.
9. Hanif R., et al., Effects of nonsteroidal antiinflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol, 1996. **52**(2): p. 237-245.
10. Elder, D.J., et al., Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti- inflammatory drug: independence from COX-2 protein expression. Clin Cancer Res, 1997. **3**(10): p. 1679-83.
11. Palayoor, S.T., et al., Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. Oncogene, 1999. **18**(51): p. 7389-94.
12. Steinauer, K.K., et al., Radiation induces upregulation of cyclooxygenase-2 (COX-2) protein in PC-3 cells. Int J Radiat Oncol Biol Phys, 2000. **48**(2): p. 325-8.
13. Folkman, J. and P.A. D'Amore, Blood vessel formation: what is its molecular basis? Cell, 1996. **87**(7): p. 1153-5.
14. Fidler, I.J., Regulation of Neoplastic Angiogenesis. J Natl Cancer Inst Monogr, 2000. **2000**(28): p. 10-14.
15. Masferrer, J.L., et al., Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res, 2000. **60**(5): p. 1306-11
16. Chiarugi, V., et al., Hypoxia induces pivotal tumor angiogenesis control factors including p53, vascular endothelial growth factor and the NFkappaB-dependent inducible nitric oxide synthase and cyclooxygenase-2. J Cancer Res Clin Oncol, 1999. **125**(8-9): p. 525-8.
17. Semenza, G.L., HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol, 2000. **88**(4): p. 1474-80.
18. Maxwell, P.H., et al., The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature, 1999. **399**(6733): p. 271-5.
19. Cockman, M.E., et al., Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem, 2000. **275**(33): p. 25733-41.
20. Ferrara, N., Vascular endothelial growth factor. Eur J Cancer, 1996. **32A**(14): p. 2413-22.
21. Kaelin, W.G., Cancer. Many vessels, faulty gene. Nature, 1999. **399**(6733): p. 203-4.

Appendices

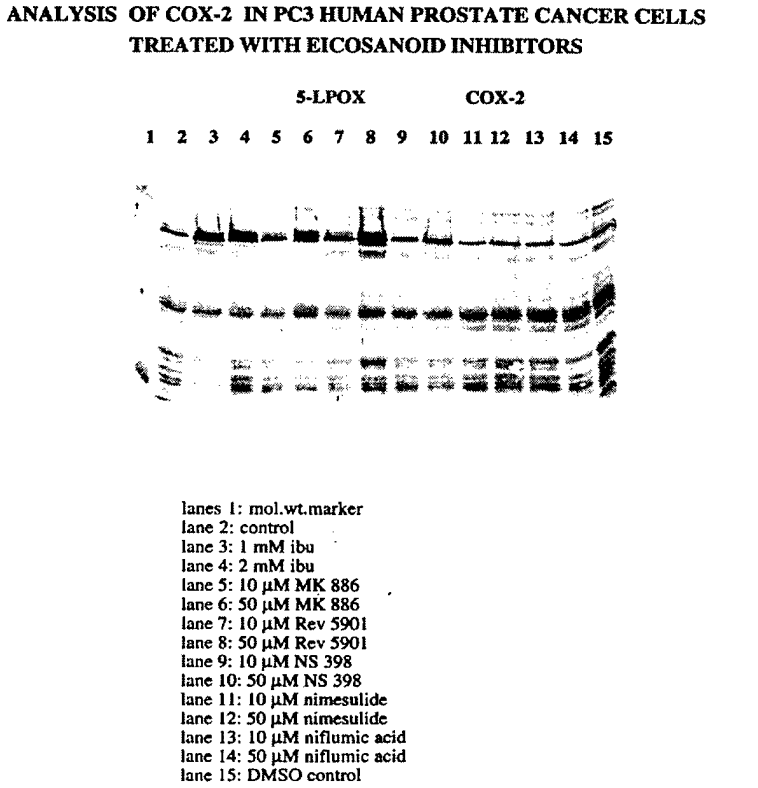
1) Fig.1: Analysis of COX-2 in PC3 human prostate cancer cells treated with eicosinoid inhibitors.

2) Abstract: S. T. Palayoor, S. K. Calderwood, E. A. Bump and C. Norman Coleman, Role of eicosanoid inhibitors in radiosensitization of PC3 prostate carcinoma cells. 47th Radiation Research society Meeting, 2000, Albuquuerque, NM.

Appendix

Fig.1

Analysis of COX-2 in PC3 human prostate cancer cells treated with eicosanoid inhibitors



Cells were treated with drugs for 24h and COX-2 protein was analyzed by western blot analysis using alkaline phosphatase-conjugated secondary antibody for detection. Although COX-2 inhibitors did not show increase in COX-2 protein at the concentrations used here, our recent data shows an increase in COX-2 protein when cells are treated with 100µM NS 398 (Figure 4).

Appendix

ABSTRACT

Role of eicosanoid inhibitors in radiosensitization of PC3 human prostate carcinoma cells. S. T. Palayoor[1], S. K. Calderwood[2], E. A. Bump[3] and C. Norman Coleman[1]. [1] National Institutes of Health, Bethesda, MD, 20892, [2]Dana Farber Cancer Institute, Boston, MA, 02115 and [3]Cleveland Clinic Foundation, Cleveland, Ohio, 44195.

Our previous studies have shown that ibuprofen, a nonsteroidal antiinflammatory agent (NSAID), enhances the effects of radiation on prostate cancer cells *in vitro* as well as *in vivo*. To determine if COX-2 was the target for the NSAID toxicity we studied the effect of ibuprofen and other NSAIDs on arachidonic acid (AA)-induced prostaglandin synthesis by ELISA and found that NSAIDs inhibited the prostaglandin synthesis at much lower concentrations than those required to induce cytotoxicity and radiosensitization. We studied the effect of higher concentrations of NSAIDs on COX-2 protein by western blot analysis. PC3 cells were found to express COX-2 constitutively. Treatment of PC3 cells with higher concentration of NSAIDs further increased COX-2 protein levels at 24h. No change in COX-2 level was observed in irradiated cells. COX-2 specific inhibitors NS398, Nimesulide and Niflumic acid were less cytotoxic and showed only slight effect on the clonogenic survival at 4 Gy. They had no effect on the constitutive COX-2 protein level. At higher concentrations NSAIDs can also inhibit lipoxygenase pathway of AA metabolism. 5-HETE, a product of AA by 5-lipoxygenase (5-LO) pathway is growth-stimulatory to PC3 cells. We studied the effect of two lipoxygenase inhibitors MK 886 and Rev 5901 on PC3 cells. Both agents were cytotoxic at micromolar concentrations and appear to enhance the effect of radiation, increasing cellular detachment, apoptosis and reducing the clonogenic cell survival. At cytotoxic concentrations they also increased COX-2 protein level. These studies suggest that lipoxygenase pathway may be a better target for the treatment of prostate cancer than the cyclooxygenase pathway. 5-LO inhibitors are clinically used in treatment of asthma, inflammation and hypersensitivity and appear to be well tolerated.

Supported by DOD Prostate Cancer Grant DAMD17-98-1-8605.

Poster presented at the 47th Radiation Research society Meeting, 2000, Albuquuerque, NM.